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Dated: October 30, 2006   Signature: _Christine Hansen_
(Christine Hansen)

Docket No.: 00131-00350-US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ann S. Robinson et al.

Application No.: 10/673000

Filed: September 26, 2003

For: USE OF HYDROSTATIC PRESSURE TO
INHIBIT AND REVERSE PROTEIN
AGGREGATION AND FACILITATE
PROTEIN REFOLDING

Confirmation No.: 9773

Art Unit: 1639

Examiner: M. C. T. Tran

DECLARATION UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Anne Skaja Robinson declare as follows:

2. I am currently an associate professor of chemical engineering at the University of Delaware and I am an inventor named on the above-referenced patent application. Attached as **Exhibit 38** is my current CV.

3. I have read the Office Action dated June 30, 2006 in the above referenced patent application.

4. I disagree with the Examiner's contention that United States Application 09/695,762, to which the above referenced application claims priority, fails to disclose the subject matter claimed in claims 3-6, 8 and 10-12. In particular I note that:

a. The "Summary of the Invention" at page 2, lines 20-28, the 09/695,762 application

teaches:

> It has been discovered that by application of hydrostatic pressure, protein aggregation can be inhibited or reversed. It is effective both in preventing aggregation during refolding and in reversing aggregation which has already taken place. After pressure is released, dissociated aggregates refold to form biologically active protein with native characteristics. ...
>
> An additional benefit of the present invention is that the **use of the present invention substantially** or even entirely obviates the need for urea or other denaturants.

(Emphasis added).

b. The summary of the invention, the 09/695,762 at page 4, lines 1-16, further states in

relevant part that:

> The present invention also provides a method to inhibit or reverse protein aggregation by subjecting a sample to high hydrostatic pressure, where the sample contains a protein aggregate, so as to substantially unfold the protein of the protein aggregate, and returning the sample to ambient pressure so as to allow the unfolded protein to refold, thereby recovering native protein from the protein aggregate. ....
>
> The invention also provides **embodiment of such methods where the sample is substantially free of a denaturing agent** selected from the group consisting of guanidine hydrochloride, guanidine thiocyanate, sodium dodecyl sulfate (SDS), and Urea. Similarly the present invention provides embodiments of such methods where the sample is substantially free of sodium dodecyl sulfate (SDS).
>
> .... Similarly, the invention provides embodiments of such methods **where the denatured protein is unfolded in the presence of a reducing agent.**

(Emphasis added).

c. One skilled in the art would necessarily understand the above passages of the

09/695,762 application as indicating that some methods of the invention involve use

of protein aggregates in a sample further containing "a chaotropic agent in an amount

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which is insufficient to denature said native protein at ambient pressure," as that

phrase is used in claims 4 and 10 of the above referenced application. Specifically,

by definition a denatured protein does not have the same conformation as the native

protein. It necessarily follows that in those embodiments of the invention which use

as a starting material a sample comprising protein aggregates and a chaotropic agent,

that it would not be possible to recover native protein at ambient pressure following

the hydrostatic pressurization step if a chaotropic agent were present in an amount

sufficient to denature the protein at ambient pressure.

5.   I disagree with the Examiner's contention that United States Application 09/695,762, to

which the above referenced application claims priority, fails to disclose the subject matter

claimed in claims 3, 5, 8 and 11, which all call for application of a pressure insufficient to

fully denature the protein. In particular I note that:

a.   The "Summary of the Invention" of 09/695,762 at page 4, lines 1-7, states that:

> The present invention also provides a method to inhibit or reverse
> protein aggregation by subjecting a sample to high hydrostatic pressure,
> where the sample contains a protein aggregate, so as to substantially
> unfold the protein of the protein aggregate, and returning the sample to
> ambient pressure so as to allow the unfolded protein to refold, thereby
> recovering native protein from the protein aggregate. In accordance with
> certain embodiment of such methods, the high hydrostatic pressure is from
> preferably about 1 to about 3.5 kbar or about 2.5 kbar.

b.   In the paragraph spanning pages 6-7 of 09/695,762 states in relevant part that:

> It has been discovered that by application of hydrostatic pressure,
> protein aggregation can be inhibited or reversed. .... After pressure is
> released, dissociated aggregates refold to form biologically active protein
> with native characteristics. .... The partially unfolded intermediates
> appear to preferably refold into the conformation of the native protein,
> rather than merely re-aggregating upon release of pressure.

c. As indicated at page 6, line 18 – page 7, line 3 of 09/695,762, it was well known in

art that application of hydrostatic pressure to proteins can be carried out without

denaturing the tertiary and/or secondary structure of proteins. In particular, as noted

at page 7, lines 1-3 of 09/695,762 (citing references), "secondary and tertiary

structures of proteins typically do not denature until pressures above 5 kbar."

d. Finally, at page 9, lines 3-12 of 09/695,762, it is stated that:

> Reversal of protein aggregation by the present invention is
> believed to be somewhat analogous to pressure dissociation of oligomeric
> proteins. **The chains that are dissociated by pressure are competent for
> rapid productive folding, perhaps because the secondary and tertiary
> structure is preserved.** The pressure-sensitive interfaces of aggregates
> are likely to be well-packed and solvent-excluded, suggesting that ag-
> gregation involves specific protein-protein interactions.
>
> In accordance with the present invention, a sample containing a
> protein of interest is subjected to high hydrostatic pressure. Preferably,
> the hydrostatic pressure is between about 0.5 kbar and 10 kbar, preferably
> about 1 kbar to about 3.5 kbar, most preferably about 2 to 3 kbar.

(Emphasis added.)

e. Based on the knowledge in the art as to the level of pressure required to denature the

secondary and tertiary structures of proteins (i.e., above 5 kbar), one skilled in skilled

in the art would immediately appreciate that the range of hydrostatic pressures

applied in some preferred embodiments (i.e., 1-3.5 kbar) would be "insufficient to

fully denature said protein" or "said protein folding intermediates."

f. Furthermore, as highlighted in the passage from 09/695,762 cited in paragraph d., the

present inventors believed that the phenomena underlying rapid productive refolding

by a method of the present invention was that secondary and tertiary structure of a

protein was preserved even during application of elevated hydrostatic pressure.

Because a "fully denatured" protein only retains primary structure, one skilled in the

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art would have necessarily understood that the inventors were describing a method wherein protein aggregates were exposed to hydrostatic pressures in range of pressure which is insufficient to fully denature the protein (i.e., a pressure at which tertiary and/or secondary structure is retained).

6. I disagree with the Examiner's contention, regarding claims 1-6 lacking written description support, that exemplification of tailspike protein aggregates using the claimed methods, which the Examiner characterizes as a "a well-known protein in which the intermediate forms of the folding and aggregates pathway of the protein are known" is insufficiently representative of the claimed method to "to demonstrate that *applicant had possession of the full scope of the claimed invention.*" In particular, I disagree with the Examiner's contention that protein aggregates of tailspike folding intermediates would not convince a skilled artisan that protein aggregates of other proteins could be used in the method. I believe that the Examiner has overlooked the knowledge in the art as of October 25, 1999, the earliest date for which priority of the current application is claimed.

7. By 1998, the issue of protein aggregation as a topic of serious study in the protein folding art had already been been "elevated to almost cult status." **Exhibit 29, p. 5253, col. 2, 2nd full paragraph.** In particular, as detailed below, it was recognized in the art that: (1) protein aggregates comprise proteins having partially denatured conformations (i.e., proteins having at least secondary structure); (2) the predominant force mediating protein aggregation was intermolecular hydrophobic interactions; (3) hydrostatic pressure could be used to disrupt intermolecular interactions, and in particular, intermolecular hydrophobic interactions; and (4) proteins have an inherent tendency to refold properly if forces driving aggregation of folding intermediates are minimized or entirely disrupted.

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8. Aggregation of native protein during processing and or storage of therapeutic protein

formulations was (and still is) a common problem. However, it was well known in the art

by 1998 at the latest:

    a. That protein aggregates can arise in protein formulations as a result of exposure to

       physical stresses (for example, shaking, mixing, thermal). See **Exhibits 1-9**.

    b. That the protein aggregates were formed in this context by noncovalent

       intermolecular interaction between partially denatured proteins (i.e., the protein

       generally retains secondary structural elements). See **Exhibits 1-2, 4-6 and 8**.

    c. And that protein aggregation in this context was generally driven by hydrophobic

       intermolecular interactions. See **Exhibits 1-2, 6 and 9**.

9. Aggregation of protein during recombinant synthesis (i.e., inclusion bodies) or purification

of recombinant protein was (and still is) a common problem. In this regard, it was well

known in the art by 1998 at the latest:

    a. That aggregation is a significant intermolecular side reaction that kinetically

       competes with intramolecular protein folding. See **Exhibit 10** (p. 13587, col. 2, ¶ 2;

       p. 13590, col. 2, last paragraph); **Exhibit 11**; **Exhibit 12**, p. 17067, col. 2, 2nd full

       paragraph (stating that:

> it is a common observation that the yield of renatured protein decreases
> when the concentration of the protein to be refolded increases. Kinetic
> competition between two types of interactions (interchain and intrachain)
> occurs during the refolding of a protein. Unimolecular intrachain
> interactions largely lead to the native state, while multimolecular
> interchain interactions would be expected to increase with the
> concentration of the refolding protein and therefore lead to misfolding and
> aggregation.);

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Exhibit 13, p. 159, col. 1, 1st full paragraph; Exhibit 14, p. 5483, col. 1, 3rd full

paragraph; and Exhibit 15, p. 3457, col. 1, end of 2nd full paragraph.

b. That protein aggregates (including inclusion bodies) generally comprise non-

covalently associated protein folding intermediates. See Exhibit 10, p. 13587, col. 2,

¶ 2 and p. 13590, col. 2, 2nd full paragraph and last paragraph; Exhibit 12; Exhibits

19-25.

c. That the protein folding intermediates which form protein aggregates (including

inclusion bodies) have considerable secondary structure. Exhibit 13, p. 159, col. 2,

1st full paragraph; Exhibit 17, sentence spanning pp. 1953-1954 and p. 1958, col. 2,

1st full paragraph; and Exhibits 26-27.

d. That protein folding intermediates tend to have an overall structure which exposes

hydrophobic surfaces which possess a strong hydrophobic nature, the intermolecular

hydrophobic interactions of which drive formation of protein aggregates. Exhibit 10,

p. 13590, col. 2, 2nd full paragraph and last paragraph; Exhibit 13, p. 159, col. 2, 1st

full paragraph – p. 160, col. 2, ¶ 1; Exhibit 14, p. 5483, col. 1, 3rd full paragraph

(stating:

> It is most likely that the intermolecular association reflects specific
> interactions between hydrophobic surfaces of one partially folded
> molecule with those of another, where these specific interactions are ones
> that normally occur intramolecularly and lead to the formation of the
> native state.... In other words if we consider the monomeric native state to
> arise by the coalescence of structural building blocks (subdomains) in an
> intramolecular fashion, the aggregates arise by these same interactions but
> in an intermolecular fashion.);

Exhibit 16; Exhibit 17, p. 1958, col. 1, ¶ 1, last sentence; Exhibit 18; Exhibit 23;

Exhibit 28, p. 428, col. 1, ¶ 2 (stating:

It is now clear, based on investigations of transient and equilibrium intermediates in vitro, that partially folded intermediates, as found with newly synthesized proteins in the cell, are particularly prone to aggregate, probably via specific intermolecular interactions between hydrophobic surfaces of structural subunits. The intermediates are more prone to aggregate than the unfolded state because in the latter the hydrophobic side chains are scattered relatively randomly in many small hydrophobic regions, whereas in the partially folded intermediates, there will be large patches of contiguous surface hydrophobicity that will have a much stronger propensity for aggregation. The tendency of partially folded intermediates to associate or aggregate is exacerbated as the protein concentration increases. The growing recognition of the critical importance of protein aggregation has resulted in a number of reviews.

(citations omitted)).

10. Regarding the use of hydrostatic pressure to dissociate proteins from protein aggregates, as of the earliest date for which priority of the current application is claimed it was already recognized in the art by 1998 at the latest that:

   a. Noncovalent interactions within a protein and between proteins can be reversibly perturbed using high hydrostatic pressure since protein folding and protein-protein interactions are normally accompanied by an increase in volume because of the combined effects of the formation of solvent-excluding cavities and the release of bound solvent. **Exhibit 30**, p. 9050, col. 2, ¶ 3; **Exhibit 35**, p. 1552, paragraph spanning columns 1-2 (stating:

   > Our conceptual framework for pressure denaturation is as follows: the protein interior is largely composed of efficiently packed residues, more likely hydrophobic than those at the surface (19). Increasing hydrostatic pressure then forces water molecules into the protein interior, gradually filling cavities, and eventually breaking the protein structure apart.).

   b. That subjection to elevated hydrostatic pressure causes dissociation of subunits of a multisubunit protein by interfering with hydrophobic interactions present in the

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contacts between the subunits. **Exhibits 31-34.** See also p. 7, lines 18-22 of the current application.

c. That proteins subjected to elevated hydrostatic pressure can still retain elements of structural organization (i.e., secondary, and sometimes tertiary structure). **Exhibit 35,** p. 1552, col. 1, last paragraph. See also p. 7, line 23 – p. 8, line 3 of the current application.

11. Finally, it was already recognized in the art by 1998 at the latest that minimizing intermolecular interactions that lead to aggregation of partially folded intermediates can be an important factor in optimizing successful refolding. **Exhibit 10,** p. 13587, col. 2, ¶ 2 and p. 13590, col. 2, last paragraph; **Exhibit 12,** p. 17067, col. 2, 2nd full paragraph; **Exhibit 13,** p. 160, col. 1, last paragraph – col. 2, 2nd paragraph; and **Exhibit 18.** Indeed, as stated by Dobson, "Many studies have established that the vast majority of denatured protein chains are capable of refolding spontaneously to the correctly folded conformation in the absence of either other macromolecules or energy expenditure." **Exhibit 29,** p. 5251, col. 1, 1st paragraph after abstract.

12. In view of art recognized common attributes of protein aggregates in general (i.e., that they are comprised of incompletely folded protein predominantly driven to associate by hydrophobic intermolecular associations (see ¶¶ 8 and 9, above)), a skilled artisan would expect that elevated hydrostatic pressure could be used to dissociate protein from aggregates of other proteins, like the dissociation of protein from tailspike protein aggregates as exemplified in the current application.

13. Moreover, a skilled artisan would also be convinced that native protein could be recovered from protein which dissociates from protein aggregates subjected to hydrostatic pressure,

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similar to the native tailspike recovered following application of hydrostatic pressure to

tailspike protein aggregates, since the phenomena underlying the ability of a dissociated

protein to assume a native conformation by the hydrostatic pressure methods of the present

invention is no different than the phenomena relied upon by every other method of

recovering native protein from protein aggregates (i.e., an inherent ability of incompletely

folded protein to spontaneously refold to a native conformation).

14. In view of the teachings found in the application and the knowledge in the art as of October

    25, 1999, it is my opinion that only routine experimentation would be required to determine

    whether protein aggregates of any particular protein are amenable for use in the currently

    claimed methods.

15. I note that the Examiner's discussion of the guidance set forth in the application as only

    including exemplification of tailspike protein aggregates (page 14 of the Office Action)

    ignores the general guidance found in the application at:

    a. Page 3, lines 22-29, describing a general method for determining the optimal

       hydrostatic pressure to recover a native protein;

    b. Page 10, lines 20-29, providing guidance on use of assays for monitoring native and

       non-native conformations, with specific exemplification of the use of HPLC in

       Example 1 of the application (pp. 14-17);

    c. Page 11, lines 1-5, providing guidance on the use of reducing agents if the protein of

       interest contains post-translational modifications;

    d. Page 11, lines 6-13, providing guidance on the use of chaperones or isomerases to

       assist refolding of a protein of interest; and

  e. Page 12, ll. 15-27, providing guidance on the temperature at which hydrostatic

   pressure should be applied.

16. The Examiner's contention as to the lack of the guidance set forth in the application also

ignores the extensive knowledge in the art regarding techniques for monitoring native and

non-native conformations of proteins as evidenced by their use in the studies reported above.

Exhibits 1-35. See also, **Exhibit 36,** pp. 445-446 ("Introduction"), and **Exhibit 37,** p.

2301, paragraph spanning columns 1-2.

17. Based on the guidance provided in the specification and the knowledge in the art in October,

1999, one skilled in the art would have been able to identify conditions under which

hydrostatic pressure could be applied to cause dissociation of protein from protein

aggregates comprising proteins other than tailspike using only a limited amount of

experimentation.

18. Likewise, one skilled in the art in October, 1999 would also have been able to identify

whether native protein was recovered following return of a sample to ambient pressure using

assays, which while depending on the particular protein of interest, were conventional

techniques used by skilled artisans in October, 1999.

19. Therefore, in my opinion, a skilled artisan in October, 1999, would have considered both the

determination of the hydrostatic pressure conditions under which protein dissociates from

protein aggregates as well as the determination of the presence of native protein following

return to ambient pressure as routine experimentation. Extensive testing as to the suitability

of other recovery methods or for optimizing recovery of protein from protein aggregates

using other methods was common in the industry at that time. See **Exhibit 13,** page 161,

paragraph spanning columns 1-2.

Application No. 10/673000   Docket No.: 00131-00350-US
Response to Office Action Dated 6/30/2006

20. I hereby declare that all statements made herein of my own knowledge are true and that all

statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful false statements and the like so made

are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the

above-identified application or any patent issues thereon.

_____   Date _10/30/06_____
         Anne Skaja Robinson

Exhibit 1

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1: <u>Biopolymers.</u> 1995 Feb;35(2):201-9.   Related Articles, Links

Impact of moisture on thermally induced denaturation and decomposition of lyophilized bovine somatotropin.

Bell LN, Hageman MJ, Bauer JM.

Drug Delivery Research and Development, Upjohn Company, Kalamazoo, MI 49007.

The nonisothermal transitions of lyophilized recombinant bovine somatotropin (rbSt) as seen via differential scanning calorimetry were evaluated with respect to moisture content. The transition peak temperature of rbSt decreased with increasing moisture from 161 degrees C in the dry state to a plateau of 65 degrees C at 28% moisture, which is similar to that of rbSt in solution. Using high performance liquid chromatography, this irreversible endothermic transition consisted primarily of unfolding, hydrophobic aggregation, and some covalent modifications. In the dry state, covalent modifications, including polymerization into compounds of higher molecular weight, were more prominent, while in the presence of moisture, hydrophobic aggregation was most prominent. The irreversibility and scan rate dependence of the endothermic phenomena supports the kinetic nature of the transition rather than a simple equilibrium between globular and unfolded states. The apparent activation energy for the net transition (i.e., unfolding, hydrophobic aggregation, and covalent modifications) was 57 kcal/mol for rbSt at 9.9% moisture. The observed enthalpy of the transition increased, decreased, then approximately leveled off as a function of increasing moisture content. This can be explained by the increasingly significant contribution of the exothermic aggregation at higher moisture contents.

PMID: 7696565 [PubMed - indexed for MEDLINE]

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1: Pharm Res. 1997 May;14(5):606-12.   Related Articles, Links

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Solvent effects on the solubility and physical stability of human insulin-like growth factor I.

Fransson J, Hallen D, Florin-Robertsson E.

Department of Pharmaceutical Technology, Pharmacia & Upjohn,
Stockholm, Sweden. jonas.fransson@eu.pnu.com

PURPOSE: The solubility and physical stability of human Insulin-like
Growth Factor I (hIGF-I) were studied in aqueous solutions with different
excipients. METHODS: The solubility of hIGF-I was determined by UV-
absorption and quantification of light blocking particles. The physical
stability of hIGF-I was studied with differential scanning calorimetry (DSC)
and circular dichroism (CD) spectroscopy. RESULTS: Human IGF-I
precipitated at low temperature in the presence of 140 mM benzyl alcohol
and 145 mM sodium chloride. CD data showed that the tertiary structure of
hIGF-I during these conditions was perturbed compared to that in 5 mM
phosphate buffer. In the presence of benzyl alcohol 290 mM mannitol
stabilized hIGF-I. Sodium chloride or mannitol by themselves had no effect
on either the solubility or the tertiary structure. Benzyl alcohol was attracted
to hIGF-I, whereas sodium chloride was preferentially excluded. The
attraction of benzyl alcohol was reinforced by sodium chloride leading to
salting-out of hIGF-I. The CD-data indicated interactions of benzyl alcohol
with phenylalanine in hIGF-I. Thermal denaturation of hIGF-I occurred in
all solutions with sodium chloride, whereas mannitol or benzyl alcohol had
no effect on the thermal stability. The thermal stability of hIGF-I was thus
decreased in 145 mM sodium chloride although it was excluded from hIGF-
I. CONCLUSIONS: The self-association and thermal aggregation of hIGF-I
is driven by hydrophobic interactions. Benzyl alcohol is attracted to hIGF-I
and induces changes in the tertiary structure causing hydrophobic attraction
of the protein at low temperatures.

PMID: 9165531 [PubMed - indexed for MEDLINE]

Exhibit 3

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1: J Pharm Sci. 1994 Dec;83(12):1657-61.   Related Articles, Links

Strategies to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development.

Chen BL, Arakawa T, Hsu E, Narhi LO, Tressel TJ, Chien SL.

Department of Pharmaceutics and Drug Delivery, Amgen Inc., Thousand Oaks, CA 91320.

Recombinant human keratinocyte growth factor (rhKGF) is a fairly unstable protein, posing a challenging problem for long-term storage. During storage, the protein unfolds at relatively low temperatures and the unfolded proteins aggregate rapidly, leading to the formation of large visible precipitates. Thermal unfolding of rhKGF displays a similar pattern, i.e., unfolding is followed immediately by aggregation as the temperature is increased. As the unfolding and aggregation (precipitation) of rhKGF limit the storage life of the protein, a search for stabilizers to suppress rhKGF unfolding and aggregation has been done by examining the effects of excipients on thermal melting temperature and on the rate of protein aggregation during storage. Sulfated polysaccharides and citrate are found to be effective in increasing the melting temperature of rhKGF or preventing its aggregation. In particular, 0.5% (w/v) heparin and high molecular weight dextran sulfate, and 0.5 M citrate are highly effective, decreasing the rates of rhKGF aggregation by about 50-fold. Other negatively charged small ions, such as phosphate, also have moderate stabilizing effects on rhKGF. A mechanistic study of the aggregation pathway of rhKGF has led to a better understanding of the stabilizing effects of these molecules. Molecules which enhance rhKGF conformational stability are capable of effectively suppressing rhKGF aggregation.

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☐ 1: J Pharm Sci. 1995 Jun

Related Articles, Links

Effect of surfactants on the physical stability of recombinant human growth hormone.

Katakam M, Bell LN, Banga AK.

Department of Pharmacal Sciences, School of Pharmacy, Auburn University, AL 36849-5503, USA.

The physical stability of a human growth hormone (hGH) formulation upon exposure to air/water interfaces (with vortex mixing) and to nonisothermal stress [determined by differential scanning calorimetry (DSC)] was investigated. The effect of these stresses on the formation of soluble and insoluble aggregates was studied. The aggregates were characterized and quantified by size exclusion-HPLC and UV spectrophotometry. Vortex mixing of hGH solutions (0.5 mg/mL) in phosphate buffer, pH 7.4, for just 1 min caused 67% of the drug to precipitate as insoluble aggregates. These aggregates were noncovalent in nature. Non-ionic surfactants prevented the interfacially induced aggregation at their critical micelle concentration (cmc) for Pluronic F-68 (polyoxyethylene polyoxypropylene block polymer) and Brij 35 (polyoxyethylene alkyl ether) and above the cmc for Tween 80 (polyoxyethylene sorbitan monooleate). However, the same surfactants failed to stabilize hGH against thermal stress in DSC studies. Higher concentrations of surfactants actually destabilized hGH as evidenced by the decrease in the onset temperature for the denaturation endotherm.

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Use of poloxamer polymers to stabilize recombinant human growth hormone against various processing stresses.

Katakam M, Banga AK.

Department of Pharmacal Sciences, School of Pharmacy, Auburn
University, Alabama 36849-5503, USA.

Several processing and shipping stresses were investigated for their effect
on the physical stability of recombinant human growth hormone (rhGH).
These included exposure to air/water interfaces, adsorption to hydrophobic
surfaces, freeze-thaw cycles, and temperature. The interfacially and
thermally denatured hormone was evaluated for the presence of insoluble
and soluble aggregates by spectrophotometry and by size-exclusion
chromatography, respectively. Noncovalent aggregates were generated by
either vortexing or multiple passages through a hypodermic needle,
processes which exposed the protein to air/water interfaces. Thermal stress
also resulted in the generation of aggregates. This aggregation was reduced
or eliminated by the use of poloxamer polymers. Under the conditions
employed, filtration through some commercially available filters, exposure
to hydrophobic beads, or multiple freeze-thaw cycles did not produce any
aggregates within the limitations of the analytical procedures used. Based on
this study, Poloxamer 407 was found to be the most effective stabilizer for
rhGH for protection against interfacial and thermal stress.

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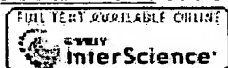
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1: J Pharm Sci. 1998 Dec;87(12):1554-9.   Related Articles, Links

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Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions.

Bam NB, **Cleland JL**, **Yang J**, **Manning MC**, **Carpenter JF**, **Kelley RF**, **Randolph TW**.

SmithKline Beecham, King of Prussia, Pennsylvania 19406, USA.

In the absence of surfactants, recombinant human growth hormone (rhGH) rapidly forms insoluble aggregates during agitation. The nonionic surfactant Tween 20, when present at Tween:protein molar ratios >4, effectively inhibits this aggregation. Differential scanning calorimetry (DSC) of rhGH solutions showed melting transitions that decreased by ca. 2 degrees C in the presence of Tween. Circular dichroism (CD) studies of the same thermal transition showed that the decrease is specific to the relatively high protein concentrations required for DSC. CD studies showed melting transitions that decreased with lower protein concentrations. Tween has an insignificant effect on the melting transition of rhGH at lower protein concentrations (0.18 mg/mL). Injection titration microcalorimetry showed that the interaction of Tween with rhGH is characterized by a weak enthalpy of binding. For comparison, interferon-g, another protein which has been shown to bind Tween, also shows weak enthalpy of binding. Fluorescent probe binding studies and infrared spectroscopic investigations of rhGH secondary structure support suggestions in the literature (Bam, N. B.; Cleland, J. L., Randolph, T. W. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol. Prog. 1996. 12, 801-809) that Tween binding is driven by hydrophobic interactions, with little perturbation of protein secondary structure.

PMID: 10189266 [PubMed - indexed for MEDLINE]

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⌐ 1: Diabetologia. 1984 Aug;27(2):212-8.   Related Articles, Links

Stabilisation of dissolved proteins against denaturation at hydrophobic interfaces.

Thurow H, Geisen K.

Studies with insulin delivery devices have shown that denaturation of dissolved proteins at hydrophobic interfaces is a basic obstacle to long-term insulin stability in pumps. This study shows that polypropylene glycol/polyethylene glycol block polymers prevent both the adsorption of dissolved proteins to hydrophobic interfaces and the resultant aggregation. At a concentration of 0.001% (w/v), the block polymer Genapol PF-10 stabilises insulin solutions over a wide range of concentrations. Insulin solutions thus stabilised are at present being clinically tested. The effectiveness of molecular variants of Genapol PF-10 to stabilise other proteins (human gamma-globulin, myoglobin and serum albumin) is presented also.

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Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII.

Kreilgaard L, Jones LS, Randolph TW, Frokjaer S, Flink JM, Manning MC, Carpenter JF.

The Department of Pharmaceutics, Royal Danish School of Pharmacy, Copenhagen.

Agitation- and freeze-thawing-induced aggregation of recombinant human factor XIII (rFXIII) is due to interfacial adsorption and denaturation at the air-liquid and ice-liquid interfaces. The aggregation pathway proceeds through soluble aggregates to formation of insoluble aggregates regardless of the denaturing stimuli. A nonionic surfactant, polyoxyethylene sorbitan monolaurate (Tween 20), greatly reduces the rate of formation of insoluble aggregates as a function of surfactant concentration, thereby stabilizing native rFXIII. Maximum protection occurs at concentrations close to the critical micelle concentration (cmc), independent of initial protein concentration. To study the mechanistic aspects of the surfactant-induced stabilization, a series of spectroscopic studies were conducted. Electron paramagnetic resonance spectroscopy indicates that binding is not occurring between Tween 20 and either the native state or a folding intermediate state of rFXIII. Further, circular dichroism spectroscopy suggests that Tween 20 does not prevent the secondary structural changes induced upon guanidinium hydrochloride-induced unfolding. Taken together, these results imply that Tween 20 protects rFXIII against freeze-thawing- and agitation-induced aggregation primarily by competing with stress-induced soluble aggregates for interfaces, inhibiting subsequent transition to insoluble aggregates.

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⌐ **1:** <u>Prikl Biokhim Mikrobiol.</u> 1979 Sep-Oct;15(5):735-9.   Related Articles, Links

[Effect of urea on aggregation of immunoglobulins in water solutions]

[Article in Russian]

Gdalevskii GIa, Kharchenko MF.

The methods of molecular light scattering and of gel filtration were used to study the degree of urea-induced desaggregation of immunoglobulin (IgG) molecules preaggregated by heating. Urea was shown to desaggregate considerably (70--80%) protein IgG aggregates. The data obtained suggest an important role of hydrophobic interactions in the immunoglobulin aggregation.

PMID: 514998 [PubMed - indexed for MEDLINE]

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Unassisted Refolding of Urea Unfolded Rhodanese*

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Station, Wilmington, Delaware 19880-0402

In vitro refolding after urea unfolding of the enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) normally requires the assistance of detergents or chaperonin proteins. No efficient, unassisted, reversible unfolding/folding transition has been demonstrated to date. The detergents or the chaperonin proteins have been proposed to stabilize folding intermediates that kinetically limit folding by aggregating. Based on this hypothesis, we have investigated a number of experimental conditions and have developed a protocol for refolding, without assistants, that gives evidence of a reversible unfolding transition and leads to >80% recovery of native enzyme. In addition to low protein concentration (10 µg/ml), low temperatures are required to maximize refolding. Otherwise optimal conditions give <10% refolding at 37 °C, whereas at 10 °C the recovery approaches 80%. The unfolding/refolding phases of the transition curves are most similar in the region of the transition, and refolding yields are significantly reduced when unfolded rhodanese is diluted to low urea concentrations, rather than to concentrations near the transition region. This is consistent with the formation of "sticky" intermediates that can remain soluble close to the transition region. Apparently, nonnative structures, *e.g.* aggregates, can form rapidly at low denaturant concentrations, and their subsequent conversion to the native structure is slow.

Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is a mitochondrial matrix enzyme which, *in vitro*, catalyzes the transfer of the outer sulfur of thiosulfate to the nucleophilic acceptor, cyanide (1, 2). This enzyme has many characteristics that make it an ideal model for studying folding processes of monomeric, multidomain proteins (3, 4). Rhodanese has a single polypeptide chain containing 293 amino acids and is folded into two, equal-sized domains (5, 6). The x-ray structure shows that no nonamino acid components are required for the native structure (5). There is no processing of the primary rhodanese translation product except for removal of the initiating methionine (7), so the folding information in the primary sequence at synthesis is still present in the isolated protein.

Rhodanese has been difficult to reactivate after unfolding in urea or guanidine hydrochloride. Under some conditions, a small fraction of unfolded rhodanese could be reactivated to a product with high specific activity (8). However, the reactivation was inefficient, and thermodynamic reversibility was not demonstrated. Aggregation was a major competition to the regain of enzyme activity.

In general, aggregation is a significant side reaction that kinetically competes with protein refolding (9-11). Therefore, minimizing intermolecular interactions that lead to aggregation of partially folded intermediates can be an important factor in optimizing successful refolding. Complete reversibility of rhodanese unfolding was achieved by including mild detergents to minimize aggregation. It was additionally necessary, in these studies, to provide reducing conditions and to include the substrate, thiosulfate (12, 13).

In a similar vein, we have recently obtained a high recovery of activity from urea-unfolded rhodanese when proteins called chaperonins were present in the refolding buffer (14). By sequestering partially folded proteins in the form of a stable binary complex, chaperonins appear to prevent aggregation by suppressing unwanted intermolecular interactions (15-17).

In order to understand better the factors that normally limit rapid, spontaneous folding, it is important to find conditions under which unassisted folding of rhodanese can be achieved. The present study focuses on optimizing spontaneous refolding from urea, and we report aspects of the unfolding/refolding processes under conditions of optimum reversibility. Rhodanese activity was used to monitor unfolding/refolding since this parameter has proven to be a sensitive indicator of subtle changes in the structure of this enzyme (3). The availability of a protocol giving reversible folding will make it possible not only to understand the folding potentials of the native protein, but also it will provide the basis for understanding the increased folding efficiency conferred by the assistance of molecular chaperones or detergents.

EXPERIMENTAL PROCEDURES

Materials—Urea was of electrophoresis purity, purchased from Bio-Rad. All other reagents were of analytical grade. Rhodanese was prepared as described previously and stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (18). Rhodanese concentration was determined using a value of $A_{280 nm}^{0.1\%} = 1.75$ (19).

Rhodanese Assay—Rhodanese activity was measured by a colorimetric method based on the absorbance at 460 nm of the complex formed between ferric ions and the reaction product, thiocyanate (19). The assay was initiated by adding microgram quantities of the enzyme, and the reaction was stopped by adding formaldehyde.

Unfolding-Refolding—For unfolding-refolding studies, rhodanese at 90 µg/ml was unfolded in 8 M urea for at least 30 min. After unfolding, the protein was diluted to 3.6 µg/ml and allowed to refold in the presence of 200 mM BME,[1] 50 mM sodium thiosulfate, and 50

[1] The abbreviation used is: BME, β-mercaptoethanol.

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mM Tris-HCl, pH 7.8. The regain of enzyme activity was used to monitor successful refolding. 30 μl of the incubating enzyme was added to 1 ml of assay mix and incubated for 10 min before determining the amount of product formed. The percent reactivation was calculated based on the activity of native enzyme that had been subjected to the refolding conditions.

Equilibrium Studies—For a typical equilibrium unfolding study, rhodanese was diluted to 3.6 μg/ml in buffers containing the indicated urea concentrations, 200 mM BME, 50 mM sodium thiosulfate, and 50 mM Tris-HCl, pH 7.8. For refolding, rhodanese at 90 μg/μl was first unfolded in 8 M urea at 24 °C. Aliquots were diluted 1:25 into buffers containing the appropriate urea concentrations to give a final protein concentration of 3.6 μg/ml and the indicated final urea concentrations.

Progress Curves—Progress curves measured the formation of the product, thiocyanate, with time. 300 μl of an enzyme solution was added to 10 ml of assay mix, and 1-ml aliquots were removed at desired times and quenched with formaldehyde, and the thiocyanate was quantified as in the standard assay above. For the nonlinear progress curves observed in some refolding experiments, the enzyme activity at any time is given by the slope at that time point.

RESULTS

Unfolding/Refolding of Rhodanese in Urea at 24 °C—The reversibility of the unfolding process was studied by monitoring enzyme activity. Activity has proven to be the most sensitive monitor of the overall integrity of the rhodanese molecule (3). For example, in detergent-assisted unfolding/refolding, the activity follows a two-state transition, and activity is the first property lost and the last property regained when the kinetics of urea-induced unfolding/folding are monitored.

There was very little reactivation when urea-unfolded rhodanese was diluted into buffer at 24 °C at a final protein concentration of 50 μg/ml, as reported previously (3). These conditions, in the presence of the detergent lauryl maltoside (5 mg/ml), give 90% reactivation. Previous studies of the detergent-assisted refolding indicated that aggregation was largely responsible for low yields of spontaneous refolding. Accordingly, we attempted to refold rhodanese at lower protein concentrations. In Fig. 1, the effects of urea on enzyme at 3.6 μg/ml are shown. Fig. 1A shows the partial reversibility of the unfolding of rhodanese at 24 °C. Some activity is regained under these conditions, but it is never more than 35%, even after 24 h. No activity was regained if the refolding was attempted at 50 μg/ml. The raised base line for the unfolding transition in Fig. 1A (▲) reflects the fact that rhodanese is directly diluted to low concentrations from the native state, and the small recovery at urea concentrations >4 M represents activity regain after dilution into the assay mix and incubation for 10 min. Initial slopes of progress curves for enzyme diluted from high urea concentrations show the initial activity is very low (see below and Ref. 20). The unfolding profile shows that the activity is almost completely lost by 3.75 M urea. To allow comparison, Fig. 1B shows the same transitions as above, except the experimental data have been normalized so that both transitions have approximately the same span. This figure shows that the curves are nearly coincident in the transition region, with a transition midpoint at 3.25 M urea.

Temperature Dependence of the Refolding of Rhodanese—Initial refolding experiments performed at 24 °C, as above, gave a maximum recovery of approximately 35%. Fig. 2 shows that spontaneous refolding of urea-unfolded rhodanese is strongly temperature-dependent. At 42 °C, very little (3%) reactivation was observed after incubation for 60 min. However, at temperatures below 42 °C the maximum yield of spontaneously reactivated rhodanese rose steadily, such that
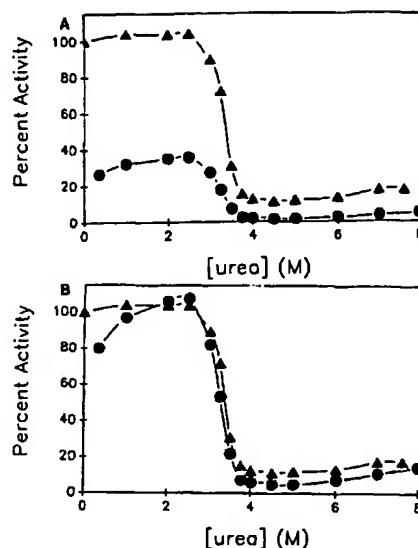

FIG. 1. **Folding transition curves of rhodanese induced by urea at 24 °C.** The unfolding/folding was followed by monitoring enzyme activity. The final concentration of the protein was 3.6 μg/ml. Activity measurements were carried out 24 h after diluting the enzyme samples to the indicated urea concentrations. *Panel A*, data obtained for unfolding (▲) and refolding (●). *Panel B*, experimental data correspond to that in *Panel A*, except that, for the refolding experiment, the data were normalized to 100% of the maximum recovery. ▲, unfolding experiments; ●, refolding experiments.
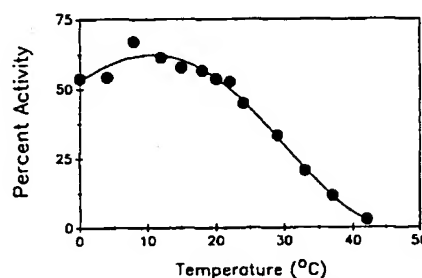

FIG. 2. **Refolding of urea-unfolded rhodanese as a function of temperature.** Urea-unfolded rhodanese was refolded as described under "Experimental Procedures" at the indicated temperatures. After 1 h, activity was measured and compared with controls containing an equal quantity of native rhodanese.

a maximum of the recoverable activity (>65%) was obtained at or below 12 °C.

Dependence of the Refolding of Rhodanese at 24 °C on the Concentration of Protein and Urea—The extent of reactivation of rhodanese shows an interdependence of the concentrations of protein and urea (Fig. 3). The concentrations of urea employed, 0–3 M, had little effect on native rhodanese (see Fig. 1). At a very low rhodanese concentration (0.3 μg/ml), the recovery was inversely dependent on the concentration of urea in the refolding buffer. For example, in 3 M urea, the recovery was 3%, but the percentage increased almost linearly when the concentration of urea was reduced. A maximum recovery of about 30% was obtained at the lowest concentration of urea (0.5 M).

A different dependence was seen at intermediate concentrations of protein (between 1–3.6 μg/ml). Here, the percentage of recovery was not very dependent on the urea concentration and ranged between 30 and 40% (Fig. 3).
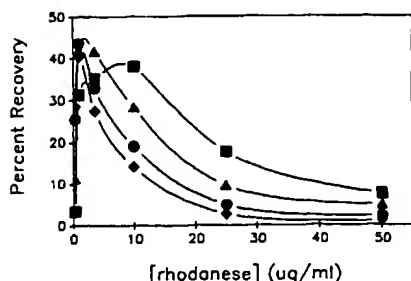
At the highest protein concentrations (>10 μg/ml), the

FIG. 3. **Effects of protein and urea concentration during rhodanese refolding at 24 °C.** Activity measurements were carried out 60 min after diluting unfolded rhodanese to the indicated urea and protein concentrations. Each *curve* represents a separate urea concentration in the refolding buffer. ♦, 0.5 M urea; ●, 1 M urea; ▲, 2 M urea; ■, 3 M urea. The final concentrations of the protein ranged from 0.3 to 50 μg/ml.
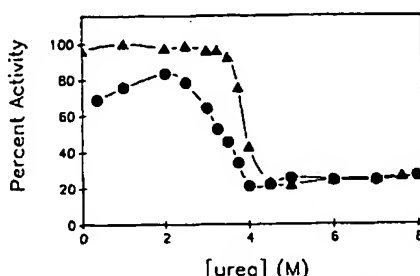

FIG. 4. **Unfolding and refolding of urea-unfolded rhodanese at 10 °C.** The experimental conditions were similar to those described under "Experimental Procedures" and in Fig. 1, but both unfolding and refolding were at 10 °C. ▲, unfolding experiments; ●, refolding experiments. Samples were incubated for 20 h after folding or unfolding prior to assay.

recovery ranged from 14 to 38%. The higher concentrations of urea gave higher recoveries of activity, the reverse of the behavior at the lowest protein concentrations. For example, at 25 μg/ml rhodanese, the recovery ranged from 1 to 18%, and maximum recovery was obtained when the concentration of urea was the highest. Finally, for a protein concentration of 50 μg/ml, the recoveries that were obtained were very low, being less than 8% at all urea concentrations in the refolding buffer, but the recoveries still increased with increasing urea concentrations.

Unfolding/Refolding of Rhodanese in Urea at 10 °C—Fig. 4 shows the reversibility of urea-induced unfolding of rhodanese at 10 °C. The process, even at such a low, near optimal temperature, does not appear to be fully reversible. The maximum recovery of activity upon refolding was approximately 80%. The maximum recovery (80%) was higher than shown in Fig. 2 (60%). However, the recoveries described in Fig. 2 were obtained after 1 h of incubation in the refolding buffer, compared with 24 h in Fig. 4. Thus, the time of incubation may be a critical parameter. The results shown in Fig. 4 (10 °C) are also distinct from those at 24 °C (Fig. 1), especially in the transition region. At 24 °C, the unfolding/refolding transitions are essentially superimposable (see Fig. 1B), but at 10 °C, curves have become separated. In comparing the two temperatures, it is seen that the unfolding transition accounts for most of the difference, and this transition appears to be shifted from 3.2 M (24 °C) to almost 4 M (10 °C). This behavior reflects slow kinetics in the transition region at the lower temperature (see below).

Kinetics of Refolding at 10 °C—To understand the apparent

nonequilibrium behavior in the unfolding/refolding transitions for rhodanese at 10 °C, kinetics were investigated. Fig. 5A shows the refolding kinetics at 10 °C. The reactivable activity that had yet to be regained was calculated using the following expression.

$$\% \text{ inactive} = 100 \times ((\% \text{ recovery})_\infty - (\% \text{ recovery})_t)/(\% \text{ recovery})_\infty$$

where $(\% \text{ recovery})_t$ is the percentage recovery of enzymatic activity at selected times, t, or 3 h (∞) after dilution of the unfolded enzyme in the refolding buffer. There was no further recovery of activity after 3 h of incubation in the refolding buffer at 24 °C. As shown, the activity was regained in a single, first-order process with a half-time of approximately 30 min.

The activity was measured as the initial slope of progress curves of the formation of product *versus* time as shown in Fig. 5B. This initial slope was taken as representative of the activity of the enzyme in the buffer just before dilution into the assay mix. Curves representing activity regain from urea concentrations higher than the transition were nonlinear, *i.e.* they showed an induction period preceding the linear steady state (Fig. 5B). This behavior has been noted previously for detergent-assisted refolding (20), and it indicates that reactivation is occurring during the assay. Linear steady state behavior from $t = 0$ was noted for samples preincubated at urea concentrations lower than the transition region (data not shown; Ref. 20).

Kinetics of Unfolding at 10 °C—Fig. 6 shows that the rate of unfolding depends on the concentration of urea. In the vicinity of the unfolding transition, unfolding can take many
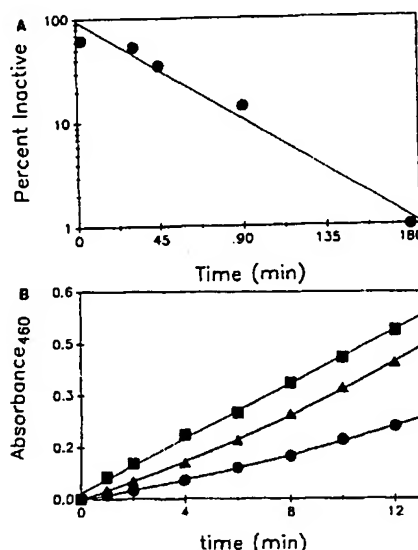

FIG. 5. **Refolding kinetics for rhodanese at 10 °C.** *Panel A*, a semilogarithmic plot of refolding kinetics of urea-unfolded rhodanese at 10 °C in 3 M urea. The final concentration of the protein was 3.6 μg/ml. The activities were corrected for the reactivation of rhodanese in the assay mix. The "percent inactive enzyme," which is capable of reactivation, was calculated as described in the text. *Panel B*, progress curves for refolding urea-unfolded rhodanese at 10 °C. The ordinate represents the absorbance due to the formation of the product thiocyanate. The *abscissa* indicates the length of time between dilution of the denaturant with a refolding buffer and the start of the assay. The concentration of the protein was 3.6 μg/ml. The incubation periods in the refolding buffers were: 2.5 min (●), 31 min (▲), and 180 min (■). The assay corresponds to diluting the sample 1:33.3 in the assay mix.